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(54) Title: NEW CONJUGATED LINOLENIC ACIDS AND METHODS FOR COMMERCIAL PREPARATION, PURIFICATION AND USES

(57) Abrégé/Abstract:
This invention relates to new conjugated linolenic acids, process for preparation thereof and method of use. Thus the invention is concerned with the preparation and purification of fatty acids which are homologues of conjugated linoleic acids, from materials rich in alpha or gamma linolenic acids. The reaction produces approximately over one third of 9Z,11E,15Z-octadecatrienoic acid from α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid). Enrichment up to and over 40% is readily performed with urea crystallization. Moreover, the product can be produced in over 90% purity by simple preparative liquid chromatography. The reaction is unique in that the reaction produces the abovementioned conjugated trienoic acid with a high selectivity, in a short time period and in relatively mild conditions.

NEW CONJUGATED LINOLENIC ACIDS AND METHODS FOR COMMERCIAL PREPARATION, PURIFICATION AND USES

FIELD OF THE INVENTION

This invention relates to new conjugated linolenic acids, process for preparation thereof and method of use. Thus the invention is concerned with the preparation and purification of fatty acids which are homologues of conjugated linoleic acids, from materials rich in alpha or gamma linolenic acids. The reaction produces approximately over one third of 9Z,11E,15Z-octadecatrienoic acid from α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid). Enrichment up to and over 40% is readily performed with urea crystallization. Moreover, the product can be produced in over 90% purity by simple preparative liquid chromatography. The reaction is unique in that the reaction produces the abovementioned conjugated trienoic acid with a high selectivity, in a short time period and in relatively mild conditions.

BACKGROUND OF THE INVENTION

Processes for the conjugation of the double bonds of polyunsaturated unconjugated fatty acids have found their main application in the field paints and varnishes. Oils comprised of triglycerides of conjugated fatty acids are known as drying oils. Drying oils have value because of their ability to polymerize or "dry" after they have been applied to a surface to form tough, adherent and abrasion resistant films. Tung oil is an example of a naturally occurring oil containing significant levels of fully conjugated fatty acids. Because tung oil is expensive for many industrial applications, research was directed towards finding substitutes.

In the 1930's, it was found that conjugated fatty acids were present in oil products subjected to prolonged saponification, as originally described by Moore, J. Biochem., 31: 142 (1937). This finding led to the development of several alkali isomerization processes for the production of conjugated fatty acids from various sources of polyunsaturated fatty acids.

The positioning of the double bonds in the hydrocarbon chain is typically not in a conjugated, i.e., alternating double bond-single bond-double bond, manner. For

example, α-linolenic acid is an eighteen carbon acid with three double bonds (18:3) at carbons 9, 12 and 15 in which all three double bonds have in the cis configuration, i.e., 9Z,12Z,15Z-C18:3 acid, γ-Linolenic acid is 6Z,9Z,12Z-C18:3 acid. Linoleic acid is 9Z,12Z-C18:2 acid (see TABLE1).

TABLE 1

Nu	Fatty Acid	Trivial Name	Structure
1	9Z,12Z,15Z-C18:3	α-Linolenic Acid	
2	6Z,9Z,12Z-C18:3	γ-Linolenic Acid	
3	9Z,12Z-C18:2	Linoleic Acid	

Migration of double bonds (e.g., leading to conjugation) gives rise to many positional and geometric (i.e., cis-trans) isomers.

Conjugated double bonds means two or more double bonds which alternate in an unsaturated compound as in 1,3 butadiene. The hydrogen atoms are on the same side of the molecule in the case of *cis* structure. The hydrogen atoms are on opposite sides of the molecule in the case of *trans* structure.

Conjugated linoleic acid (CLA) is a general term used to name positional and geometric isomers of linoleic acid. Linoleic acid is a straight chain carboxylic acid having double bonds between the carbons 9 and 10, and between carbons 12 and 13. For example, one CLA positional isomer has double bonds between carbons 9 and 10 and carbons 11 and 12 (i.e, 9Z,11E-C18:2 acid); another has double bonds between carbons 10 and 11 and carbons 12 and 13 (i.e., 10E,12Z-C18:2 acid), each with several possible cis and trans isomers.

TABLE 2

Nu	Fatty Acid	Trivial Name	Structure
	9Z,11E-C18:2	Rumenic Acid	
	10E,12Z-C18:2	none	

The 9Z,11E-C18:2 isomer has been shown to be the first intermediate produced in the biohydrogenation process of linoleic acid by the anaerobic rumen bacterium *Butyrvibrio fibrisolvens*. This reaction is catalyzed by the enzyme linoleate isomerase which converts the cis-12 double bond of linoleic acid into a *trans*-11 double bond. (C. R. Kepler et al., 241 J. Biol. Chem. (1966) 1350). It has also been found that the normal intestinal flora of rats can also convert linoleic acid to the 9Z,11E-C18:2 acid isomer. The reaction does not, however, take place in animals lacking the required bacteria. Therefore, CLA is largely a product of microbial metabolism in the digestive tract of primarily ruminants, but to a lesser extent in other mammals and birds.

The free, naturally occurring conjugated linoleic acids (CLA) have been previously isolated from fried meats and described as anticarcinogens by Y. L Ha, N K. Grimm and M. W. Pariza, in Carcinogenesis, Vol. 8, No. 12, pp. 1881-1887 (1987). Since then, they have been found in some processed cheese products (Y. L. Ha, N. K. Grimm and M. W. Pariza, in J. Agric. Food Chem., Vol. 37, No. 1, pp. 75-81 (1987)). Cook et al. in U.S. Pat. No. 5,554,646 disclose animal feeds containing CLA, or its non-toxic derivatives, e.g., such as sodium and potassium salts of CLA, as an additive in combination with conventional animal feeds or human foods. CLA makes for leaner animal mass.

The biological activity associated with CLAs is diverse and complex (Pariza et al. in Prog. Lipid Research., Vol 40, pp. 283-298).

Anti-carcinogenic properties have been well documented, as well as stimulation of the immune system. Administration of CLA inhibits rat mammary tumorogenesis, as demonstrated by Ha et al., Cancer Res., 52:2035-s (1992). Ha et al., Cancer Res., 50:1097 (1990), reported similar results in a mouse forestomach neoplasia model. CLA has also been identified as a strong cytotoxic agent against target human melanoma, colorectal and breast cancer cells in vitro. A recent major review article confirms the conclusions drawn from individual studies (Ip, Am. J. Clin. Nutr. 66(6):1523s (1997)). In *in vitro* tests, CLAs were tested for their effectiveness against the growth of malignant human melanomas, colon and breast cancer cells. In the culture media, there was a significant reduction in the growth of cancer cells treated with CLAs by comparison with control cultures. The mechanism by which

CLAs exert anticarcinogenic activity is unknown. In addition, CLAs have a strong antioxidative effect so that, for example, peroxidation of lipids can be inhibited (Atherosclerosis 108, 19-25 (1994)). U.S. Pat. No. 5,914,346 discloses the use of CLA's to enhance natural killer lymphocyte function. U.S. Pat. No. 5,430,066 describes the effect of CLA's in preventing weight loss and anorexia by immune system stimulation.

Although the mechanisms of CLA action are still obscure, there is evidence that some component(s) of the immune system may be involved, at least in vivo. U.S. Pat. No. 5,585,400 (Cook, et al., incorporated herein by reference), discloses a method for attenuating allergic reactions in animals mediated by type I or IgE hypersensitivity by administering a diet containing CLA. CLA in concentrations of about 0.1 to 1.0 percent was also shown to be an effective adjuvant in preserving white blood cells. U.S. Pat. No. 5,674,901 (Cook, et al., incorporated herein by reference), disclosed that oral or parenteral administration of CLA in either free acid or salt form resulted in elevation in CD-4 and CD-8 lymphocyte subpopulations associated with cell mediated immunity. Adverse effects arising from pretreatment with exogenous tumor necrosis factor could be alleviated indirectly by elevation or maintenance of levels of CD-4 and CD-8 cells in animals to which CLA was administered.

CLA's have also been found to exert a profound generalized effect on body composition, in particular, upon redirecting the partitioning of fat and lean tissue mass. U.S. Pat. Nos. 5,554,646 and 6,020,378 disclose the use of CLA's for reducing body fat and increasing lean body mass. U.S. Pat. No. 5,814,663 discloses the use of CLA's to maintain an existing level of body fat or body weight in humans. U.S. Pat. No. 6,034,132 discloses the use of CLA's to reduce body weight and treat obesity in humans. CLA's are also disclosed by U.S. Pat. No. 5,804,210 to maintain or enhance bone mineral content. EP 0 579 901 B relates to the use of CLA for avoiding loss of weight or for reducing increases in weight or anorexia caused by immunostimulation in human beings or animals. U.S. Pat. No. 5,430,066 (Cook, et al., incorporated herein by reference), describes the effect of CLA in preventing weight loss and anorexia by immune stimulation.

CLA has been found to be an in vitro antioxidant, and in cells, it protects membranes from oxidative attack. In relation to other important dietary antioxidants, it quenches singlet oxygen less effectively than beta.-carotene but more effectively than .alpha.-tocopherol. It appears to act as a chain terminating antioxidant by chain-propagating free radicals (U.S. Pat. No. 6,316,645 (Sih, et al., incorporated herein by reference)).

Skin is subject to deterioration through dermatological disorders, environmental abuse (wind, air conditioning, central heating) or through the normal aging process (chronoaging) which may be accelerated by exposure of skin to sun (photoaging). In recent years the demand for cosmetic compositions and cosmetic methods for improving the appearance and condition of skin has grown enormously. WO 95/13806 discloses the use of a composition comprising zinc salts of 68% (unconjugated) linoleic acid and 10% conjugated isomers of linoleic acid for use in treating skin disorders.

Apart from potential therapeutic and pharmacologic applications of CLA as set forth above, there has been much excitement regarding the use of CLA as a dietary supplement. CLA has been found to exert a profound generalized effect on body composition, in particular redirecting the partitioning of fat and lean tissue mass. U.S. Pat. No. 5,554,646 (Cook, et al., incorporated herein by reference), discloses a method utilizing CLA as a dietary supplement in which pigs, mice, and humans were fed diets containing 0.5 percent CLA. In each species, a significant drop in fat content was observed with a concomitant increase in protein mass. It is interesting that in these animals, increasing the fatty acid content of the diet by addition of CLA resulted in no increase in body weight, but was associated with a redistribution of fat and lean within the body. Another dietary phenomenon of interest is the effect of CLA supplementation on feed conversion. U.S. Pat. No. 5,428,072 (Cook, et al., incorporated herein by reference), provided data showing that incorporation of CLA into animal feed (birds and mammals) increased the efficiency of feed conversion leading to greater weight gain in the CLA supplemented birds and mammals. The potential beneficial effects of CLA supplementation for food animal growers is apparent.

Another important source of interest in CLA, and one which underscores its early commercial potential, is that it is naturally occurring in foods and feeds consumed by

humans and animals alike. In particular, CLA is abundant in products from ruminants. For example, several studies have been conducted in which CLA has been surveyed in various dairy products. Aneja, et al., (J. Dairy Sci., 43: 231 [1990]) observed that processing of milk into yogurt resulted in a concentration of CLA. Shanta, et al. (Food Chem., 47: 257 [1993]) showed that a combined increase in processing temperature and addition of whey increased CLA concentration during preparation of processed cheese. In a separate study, Shanta, et al., J. Food Sci., (60: 695 [1995]) reported that while processing and storage conditions did not appreciably reduce CLA concentrations, they did not observe any increases. In fact, several studies have indicated that seasonal or interanimal variation can account for as much as three fold differences in CLA content of cows milk (Parodi, et al., J. Dairy Sci., 60: 1550 [1977]). Also, dietary factors have been implicated in CLA content variation (Chin, et al., J. Food Comp. Anal., 5: 185 [1992]). Because of this variation in CLA content in natural sources, ingestion of prescribed amounts of various foods will not guarantee that the individual or animal will receive the optimum doses to ensure achieving the desired nutritive effect.

Economical conjugated fatty acid production in commercial quantities for use in domestic food animal feeds is a desirable objective in light of the nutritional benefits realized on a laboratory scale. Preferably, the conjugated fatty acid is produced directly from a source of raw vegetable oil and not from expensive purified linoleic acid. Further, the process must avoid cost generating superfluous steps, and yet result in a safe and wholesome product palatable to animals.

All the useful methodologies for preparation of conjugated linoleic acid (CLA) were recently reviewed by Adlof (In:Yurawecz *et al.* (Ed), Advances in Conjugated Linoleic Acid Research, volume 1, AOCS Press, Champaign, Il, pp 21-38 [1999]).

The usual methodology for conjugation of polyunsaturated fatty acids is alkali-catalyzed isomerization. This reaction may be performed using different bases such as hydroxides or alkoxides in solution in appropriate alcoholic reagents. This reaction was developed in the 1950's for spectrophotometric estimation of polyunsaturated fatty acids in fats and oils [AOCS official method Cd 7-58; JAOCS 30:352 (1953)].

In alkali isomerization the fatty acids are exposed to heat, pressure and a metal hydroxide or oxide in nonaqueous or aqueous environments, resulting in the formation of conjugated isomers. Other methods have been described which utilize metal catalysts, which is not as efficient in the production of conjugated double bonds. It was found that isomerization could be achieved more rapidly in the presence of higher molecular weight solvent. Kass, et al., J. Am. Chem. Soc., 61: 4829 (1939) and U.S. Pat. No. 2,487,890 (1950) showed that replacement of ethanol with ethylene glycol resulted in both an increase in conjugation in less time. U.S. Pat. No. 2,350,583 and British Patent No. 558,881 (1944) achieved conjugation by reacting fatty acid soaps of an oil with an excess of aqueous alkali at 200-230 degrees C. and increased pressure.

Dehydration of methyl ricinoleate (methyl 12-hydroxy-*cis*-9-octadecenoate) (Gunstone and Said, Chem. Phys. Lipids 7, 121 [1971]; Berdeaux et al., JAOCS 74, 1011 [1997] give 9Z,11E-C18:2 isomer as a major product. U.S. Pat. Nos. 5,898,074 disclosed a synthesis process for producing this fatty acid at room temperature in high yield. The tosylate or the mesylate of the methyl ester of ricinoleic acid is formed with tosyl chloride or mesyl chloride in a pyridine solvent or in acetonitrile and triethyl amine. The obtained tosylate or mesylate is reacted with diazabicyclo-undecene in a polar, non-hydoxylic solvent of acetonitrile to form the preferred isomer of 9c,11t-18:2 methyl ester in high yield. U.S. Pat. Nos. 6,160,141 disclosed a synthesis process for producing conjugated eicosanoid fatty acid from methyl lesquerolate (methyl 14-hydroxy-*cis*-11-octadecenoate) at room temperature in high yield using the same principle.

Among the processes known to effect isomerization without utilizing an aqueous alkali system, is a nickel-carbon catalytic method, as described by Radlove, et al., Ind. Eng. Chem.38: 997 (1946). A variation of this method utilizes platinum or palladium-carbon as catalysts. Conjugated acids may also be obtained from α-hydroxy allylic unsaturated fatty acid using acid catalyzed reduction (Yurawecz et al., JAOCS 70, 1093 [1993]), and partial hydrogenation of conjugated acetylenic acid such as santalbic (11E-octadec-9-ynoic) acid using Lindlar's catalyst could also be used but are limited by natural sources of such fatty acid.

Natural fully conjugated linolenic acids (CLNA) have been found at high content levels in some seed oils (Hopkins, In:Gunstone, F.D. (Ed), Topics in Lipid Chemistry, volume 3, ELEK Science, London, pp 37-87 [1972]). For example, Takagi and Itabashi (Lipids 16, 546 [1981]) reported calendic acid (8E,10E,12Z-C18:3 acid, 62.2%) in pot marigold seed oil, punicic acid (9Z,11E,13Z-C18:3 acid, 83.0%) in pomegranate seed oil, α-eleostearic acid (9Z,11E,13E-C18:3 acid) in tung (67.7%) and bitter gourd (56.2%) seed oils, and catalpic acid (9E,11E,13Z-C18:3 acid, 42.3%) in catalpa seed oil, respectively.

An octadecatrienoic acid isomer whose structure has been tentatively defined as 9Z,11E,15Z-C18:3 acid, is believed to be the first intermediate in the biohydrogenation process of α-linolenic acid by the anaerobic rumen bacterium *Butyrvibrio fibrisolvens* (C. R. Kepler and S. B. Tove 242 J. Biol. Chem. (1967) 5686).

SUMMARY OF THE INVENTION

The present invention relates to to new conjugated linolenic acids, process for preparation thereof and method of use. In a general sense, the invention describes a process for preparation and purification of fatty acids which are homologues of conjugated linoleic acids from natural and/or synthetic materials rich in alpha or gamma linolenic acids. In a prefered embodiment the reaction produces approximatively over one third of 9Z,11E,15Z-C18:3 acid from alpha linolenic acid (9Z,12Z,15Z-C18:3 acid). In a second embodiment, enrichmnent up to and over 40% is readily performed with urea cristallization. Moreover, the product can be produced in over 90% purity by simple preparative liquid chromatography. In another embodiment, the products obtained include free fatty acids, and derivatives thereof, including, but not limited to esters, amides, salts, fatty alcohols. The reaction is unique in that the reaction produces the abovementioned conjugated trienoic acid with a high selectivity, in a short time period and in relatively mild conditions.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 presents mass spectra of produtcs resulting of the isomerization process of alpha-linolenic acid (9Z,12Z,15Z-C18:3 acid), as 4,4-dimethyloxazoline derivatives:

A, 9Z,11E,15Z-C18:3; B, 9,11,13-C18:3, C, 10E,12Z,14E-C18:3 and D, 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid);

Fig. 2 present the thermal mechanism leading to the formation of 11,13-CCLA [9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid (Fig 1-D)] from 10E,12Z,14E-C18:3 acid.

Fig. 3 present gas liquid chromatograms of fatty acid methyl esters obtained after methylation of linseed oil (A), conjugated linseed oil (B) liquid phase from urea crystallization (C), reversed-phase liquid chromatography fraction containing about 97 % of 9Z,11E,15Z-C18:3 acid (D), argentation liquid chromatography fraction containing about 99+ % of 9Z,11E,15Z-C18:3 acid (E);

Fig. 4 presents the gas liquid chromatogram of the fatty acid methyl esters obtained after methylation of partially conjugated evening primrose oil.

DETAILED DESCRIPTION OF THE INVENTION

The oils and fats, alone or as mixtures, containing alpha-linolenic acid may include but are not limited to arnebia, basil, candelnut, flax (linseed), linola, gold of pleasure, hemp, mustard, perilla, soybean, canola, walnut, chia, crambe, echium, hop, kiwi, pumkin and purslane seed oils, or any other oil, wax, ester or amid that is rich in linolenic acid.

The oils and fats, alone or as mixtures, containing gamma-linolenic acid may include but are not limited to borage, evening primrose and black currant seed oils, or any other oil, wax, ester or amid that is rich in linolenic acid.

The disclosed process converts double bonds of α- and γ-linolenic acid isomers into partly and/or fully conjugated systems as well as into cyclic fatty acid isomers. The process which can be performed both in batch and continuous modes, involves blending one or a mixture of vegetable oils with various concentration of linoleic acid or partial glycerides of such oils, or partially purified or concentrated isomers with 0.5 to 10 moles of base such as sodium hydroxide, sodium alkoxylate, sodium metal, potassium hydroxide, potassium alkoxylate, potassium metal, and strong base resins. The reaction proceeds at temperatures from 20°C up to 280°C in the solvent, selected from commercial polyols such as propylene glycol, glycerol and

ethylene glycol, for periods of 30 sec to 18 hours, depending on the base and/or the temperature and/or solvent, and/or substrate and/or a desire expected conversion rate. After cooling, if required, to 20-80°C, acid is added to the reaction mixture to neutralize the soaps and/or remaining base in the reactor. It is preferred to bring the pH of the contents of the reactor to pH4 or less through the addition of either a mineral or organic acid. Acids that may be used include, but are not limited to, hydrochloric acid, sulfuric acid, phosphoric acid and citric acid. The solvent phase (polyol + water) is withdrawn and the remaining fatty acid rich phase can be washed with water and/or saline solutions of variable concentration such as sodium chloride (5%w/w) to remove traces of acids used for acidification of the reaction mixture. Remaining water can be removed by usual means (i.e. centrifugation, vacuum, distillation of drying agents). As described in example 1, the concentration of 9Z,11E,15Z-C18:3 acid in the product is approximately 33%. This product, as such or converted into derivatives, can be used in nutrition, cosmetic, nutraceutical, biological and/or animal feed applications.

Isomer composition of the formed fatty acid was determined by gas-liquid chromatography coupled with a mass-spectrometer (GC-MS) of their 4,4-dimethyloxazoline (DMOX) derivatives. The use of derivatives is a necessary step prior to structural determination of fatty acid by GC-MS because mass spectra of fatty acid methyl ester, the usual derivatives for gas-liquid chromatography analysis, are devoid of sufficient information for identification of structural isomers. This is mainly due to the high sensitivity of the carboxyl group to fragmentation and to double bond migration (Christie, W.W., Gas Chromatography–Mass Spectrometry Methods for Structural Analysis of Fatty Acids, Lipids 33:343–353 (1998).). However, stabilization of the carboxyl group by the formation of a derivative containing a nitrogen atom results in mass spectra that allow structural determination for most fatty acids. Indeed, these fatty acids derivatives provide diagnostic fragments that allow accurate structure determination. The derivatives were submitted to GC-MS with a Hewlett Packard 5890 Series II plus gas chromatograph attached to an Agilent model 5973N MS Engine. The latter was used in the electron impact mode at 70 eV with a source temperature of 230.degree. C. The GC was fitted with split injection. For DMOX derivatives a open tubular capillary column coated with BPX-70 (60 m.times.0.25 mm, 0.25 µm film; SGE, Melbourne,

Australia) was used. After holding the temperature at 60.degree. C. for 1 min, the oven temperature was increased by temperature-programming at 20.degree. C./min to 170.degree. C where it was held for 30 min., then at 5.degree. C./min to 210.degree. C. where it was held for 30 min. Helium was the carrier gas at a constant flow-rate of 1 mL/min, maintained by electronic pressure control.

Mass spectrum of conjugated products of 9Z,12Z,15Z-C18:3 acid obtain by conjugation of linseed oil were presented in FIG. 1.

Structural formula and mass spectrum of the DMOX derivatives of the 9Z,11E,15Z-C18:3 acid are illustrated in FIG. 1A. The DMOX has a molecular ion at m/z=331, confirming the octadecatrienoic acid structure. The ion at m/z=262 confirms the location of the 11,15-double bond system (by extrapolation from the known 5,9-isomer (Berdeaux and Wolff, J. Am. Oil Chem. Soc., 73: 1323-1326 (1996)), and gaps of 12 a.m.u. between m/z=208 and 196, and 288 and 276 verify the location of double bonds in positions 9 and 15, respectively. Mass spectrometry does not confirm the geometry of the double bonds, but they have been determined according to Nichols et al. (J. Am. Chem. Soc, 73:247-252 (1951)) based on the Ingold theory on the prototropic shift mechanism (Ingold, J. Chem. Soc, 1477 (1926)).

Structural formula and mass spectrum of the DMOX derivatives of the 9,11,13-C18:3 acid are illustrated in FIG. 1B. The DMOX has a molecular ion at m/z=331, confirming the octadecatrienoic acid structure. Gaps of 12 a.m.u. between m/z=208 and 196, and 222 and 234, and 248 and 260 verify the location of double bonds in positions 9 ,11 and 13, respectively. Four different minor isomers of 9,11,13-C18:3 are present in the reaction products. The most abundant is the 9Z,11Z,13E-C18:3 acid isomer which is known as α-eleostearic acid.

Structural formula and mass spectrum of the DMOX derivatives of the 10E,12Z,14E-C18:3 acid are illustrated in FIG. 1C. The DMOX has a molecular ion at m/z=331, confirming the octadecatrienoic acid structure. Gaps of 12 a.m.u. between m/z=210 and 222, and 236 and 248, and 262 and 274 verify the location of double bonds in positions 10 ,12 and 14, respectively. Mass spectrometry does not confirm the geometry of the double bonds, but they have been determined according to Nichols et al. (J. Am.

Chem. Soc, 73:247-252 (1951)) based on the Ingold theory on the prototropic shift mechanism (Ingold, J. Chem. Soc, 1477 (1926)). The 10E,12Z,14E-C18:3 acid isomer is prone to cyclization, thus forming cyclohexadienyl compound (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid)) by an electrocyclization process presented in FIG. 2.

Structural formula and mass spectrum of the DMOX derivatives of the 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid) are illustrated in FIG. 1D. The DMOX has a molecular ion at m/z=330 –1, confirming the occurrence of a high stabilized conjugated ion fragment (radical in carbon 10 or 15, stabilized by resonance effect). A distinctive ion at m/z=288 is characteristic for alpha cleavage occurring in cyclic fatty acids (Sébédio at al. J. Am. Oil Chem. Soc., 64: 1324-1333 (1987)). The gap of 78 atomic mass units (a.m.u.) between m/z=288 and 210 is that expected for the cyclohexadienyl group which conjugated double bond system in positions 11 and 13.

Reaction progress was determined by gas-liquid chromatography under appropriate condition as presented in example 1.

Increasing the concentration of, for example 9Z,11E,15Z-C18:3 acid, can be achieved using different methods, alone or in combination. One method makes use of urea complexation. Urea solution, prepared at a temperature ranging from 20 to 90°C in different solvents or mixtures thereof, selected from water, and/or alcohols. Complexation is performed at the same temperature by addition of the product in a molar ratio of 0.5 to 8, and cooling at a temperature range of 30 to -30°C, as required. The above-mentioned 9Z,11E,15Z-C18:3 acid is isolated in higher concentration after treatment of the liquid phase, obtained after separation from the solid phase, by using conventional means such as filtration or centrifugation. Decomplexation is then carried out by addition of either a diluted organic or mineral acid. Acids that may be used include, but are not limited to, hydrochloric acid, sulfuric acid, phosphoric acid and citric acid. The product is obtained by decantation or liquid-liquid extraction with an organic solvent such as but not limited to hexane, heptane, petroleum ether and ligroin. If required, the organic solvent is eliminated (i.e. evaporated or distilled). A preferred description of the present embodiment is described in example 2.

Another method for raising level of, for example 9Z,11E,15Z-C18:3 acid, either as free acid or derivatized (i.e methyl, ethyl, isopropyl, butyl, phenyl) is liquid chromatography using various convenient stationary phases. One particular is reversed phase liquid chromatography (i.e. ODS) for which eluents may include but are not limited to water, acetonitrile, acetone, methanol, tetrahydrofuran, methyl-tertbutyl ether, and combination thereof. A detailed description of the method is described in example 3. Argentation liquid chromatography may be used to isolate specific isomers from a complex mixture of fatty acid ester or free fatty acid. A detailed description of the methodlogy applied to the 9Z,11E,15Z-C18:3 acid isomer is described in example 4.

Still another method for raising the concentration level of, for example 9Z,11E,15Z-C18:3 acid, either as free acid or derivatized (i.e methyl, ethyl, isopropyl, butyl, phenyl) is cristallization, either in solvent or mixture thereof, such as, but not limited to, acetone, methanol, pentane, or in absence of solvent (i.e. dry fractionation). A detailed cooling program is required in order to obtain a more concentrated product. One particular case is that of further cristallization of urea complexes of fatty acids.

The above-mentioned new products that we claim are fully characterized by the following physico-chemical properties

viscosity
refractive index
UV-absorption (max)
flash point
dropping point
peroxide value
iodine value

Dietary supplements, food additives, cosmetic and pharmaceutical compositions formulated with conjugated products of α- and γ-linolenic acid and their related compounds used individually or in mixtures with other products claim the following biological properties: inhibiting carcinogenesis; enhancing immune response; reducing effects of immune stimulation; reducing atherosclerosis; enhancing growth;

regulation of physiology of adipocyte cell; reducing subcutaneous fat; redirecting the partitioning of fat and lean tissue mass; increasing lean body mass; maintaining or enhancing bone mineral content; reducing adipocyte cell number; inhibiting lipoprotein lipase activity; reducing apolipoprotein B secretion.; regulation of lipoprotein physiology; modulating triacylglycerol, free fatty acids, cholesterol and lipoprotein metabolism; modulating nutritional components of milk produced by a lactating animal; enhancing whole body protein accretion; enhancing CPT activity in skeletal muscle; inducing changes in the regulation and/or action of Tumor Necrosis Factor-alpha (TNF-alpha); antioxidant by chain-propagating free radicals; treating skin disorders; protecting biological membranes from oxidative attack; effects on blood insulin; effect on hepatic lipid metabolism.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: kg (kilograms); g (grams); mg (milligrams); °C (degrees centigrade); L (liters); mL (milliliters); µL (microliters); m (meters); cm (centimeters); mm (millimeters), µm (micrometers); NaOH (sodium hydroxide), H_2SO_4 (sulfuric acid), NaCl (sodium chloride); 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid), $AgNO^3$ (silver nitrate).

EXEMPLE 1

Preparation of mixture containing high amount of 9Z,11E,15Z-C18:3 acid by conjugation of linseed oil

To commercial propylene glycol (46.48 kg) were added NaOH (1.94 kg) at room temperature. The resulting mixture was heated at 160°C for 20 min into a 200 L stainless steal reactor under nitrogen atmosphere and with vigorous agitation. Commercial raw linseed oil (4.19 kg) was added under nitrogen atmosphere. The mixture was heated at 160°C for 2 hours under nitrogen atmosphere and with vigorous agitation. After cooling to 80°C, the reaction mixture was directly acidified

with aqueous solution of H_2SO_4 (0.06 % w/w, 47.5 kg). After standing 10 min, the top layer was washed with a NaCl aqueous solution (5% w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

Fatty acid composition of the resulting product was performed by high resolution gas-chromatography after methylation of a sample (20 mg) using the boron trifluoride method (Metcalfe et al.). The analytical equipment consisted of an Agilent Technologies GLC 6890 with auto sampler. The column was a highly polar open tubular capillary type. The following program setting were used (TABLE 3):

TABLE 3

Injection	Split mode 1:50 at 250°C
Detection	Flame Ionization Detector at 250°C
Carrier	Helium at 249.5 KPa at 170°C
Oven Program	60°C for 1 min then 20°C.min^{-1} to 170°C and 170°C throughout for 30 min, then 5°C.min^{-1} 210°C C throughout for 5 min
Column	BPX-70 capillary column, 60 m X 0.25 mm i.d., 0.25 µm film thickness

The obtained chromatogram was given in fig 3 B. The quantitative conversion of alpha-linolenic acid was confirmed and the mixture contain approximately 33 % of 9Z,11E,15Z-C18:3, fatty acid composition of the mixture was given in table 4.

TABLE 4

Fatty Acid	% Before Reaction	% After Reaction
Palmitic	5.40	5.07
Stearic	4.13	3.20
Oleic	19.77	19.27
11Z-C18:1	0.69	0.65
Linoleic	16.47	7.16
alpha-Linolenic	53.54	0.87
9Z,11E-C18:2	0.00	4.89
10E,12Z-C18:2	0.00	4.79
11,13-CCLA	0.00	8.73
9Z,11E,15Z-C18:3	0.00	32.98
9,11,13-C18:3[1]	0.00	3.73
10E,12Z,14E-C18:3	0.00	6.06
10,12,14- C18:3[2]	0.00	1.41

[1]stereochemistry of the double bonds not identified

[2]other stereo isomers of 10,12,14-C18:3 Acid

EXEMPLE 2

Preparation of mixture containing high amount of 9Z,11E,15Z-C18:3 acid by conjugation of linseed oil and consecutive urea crystallization

The top layer (3.26 kg) obtained in example 1 was removed and transferred in a 20 L reactor containing a solution of urea (3.26 kg) in aqueous ethanol (95 %, v/v, 13.20 kg) prepared at 60°C under nitrogen condition for 1 hour. Free fatty acids were homogenized and the obtained mixture was cooled at 4°C for 12 h. The liquid phase (17.77 kg) was removed from the solid phase (3.18 kg) by centrifugation and transferred into a 100 L, stainless steal, sight glasses reactor. An aqueous solution of H_2SO_4 (0.1 %, w/w, 49.12 kg) was added to the mixture and the solution was vigorous shacked for 1 min under nitrogen atmosphere. After standing 10 min, the top layer was washed with a NaCl aqueous solution (5% w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

The solid phase (3.18 kg) was dissolved in a solution of H_2SO_4 (0.1 %, w/w, 49.12 kg) at 70°C and transferred into a 107 L, stainless steal, sight glasses reactor and the solution was vigorous shacked for 1 min under nitrogen atmosphere. After standing 10 min, the top layer was washed in the same apparatus with a NaCl aqueous solution (5% w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

Fatty acid composition of the resulting products was performed by high resolution gas-chromatography after methylation of samples (20 mg) using the boron trifluoride method (Metcalfe et al.). The used analytical conditions were the same as presented in example 1.

The obtained chromatogram was given in fig 3C and fatty acid composition of the mixture was given in table 5.

TABLE 5

Fatty Acid	% Before Crystallization	% in Liquid Phase	% in Solid Phase
Palmitic	5.07	0.58	15.41
Stearic	3.20	0.04	12.17
Oleic	19.27	17.19	27.88
11Z-C18:1	0.65	0.66	0.84
Linoleic	7.16	8.50	2.60
Alpha-Linolenic	0.87	0.79	0.17
9Z,11E-C18:2	4.89	5.86	4.17
10E,12Z-C18:2	4.79	6.21	2.59
11,13-CCLA	8.73	10.61	1.42
9Z,11E,15Z-C18:3	32.98	40.74	10.88
9,11,13-C18:3[1]	3.73	3.54	3.17
10E,12Z,14E-C18:3	6.06	0.73	13.78
10,12,14- C18:3[2]	1.41	1.26	1.72

[1]stereochemistry of the double bonds not identified

[2]other stereo isomers of 10,12,14-C18:3 Acid

EXEMPLE 3

Preparation and purification of 9Z,11E,15Z-C18:3 acid by reverse phase liquid chromatography.

Products obtained in example 1 and 2 containing a high level of 9Z,11E,15Z-C18:3 were submitted to a preparative high performance liquid chromatograph fitted with a preparative ODS (octadecylsilyl) column (25 cm X 6.5 cm i.d.). The mobile phase was methanol and water (90:10, v/v, 400 mL.min^{-1}). The sample (10 g) was injected at atmospheric pressure and the separation was achieved in 60 min. Collected fractions were analyzed by gas-liquid chromatography as presented in example 1, and a typical gas-chromatogram was presented in fig 3D. The desire compound eluted in the first partition (partition number = 12) that allow a purification of about 95%.

EXEMPLE 4

Preparation and purification of 9Z,11E,15Z-C18:3 acid by argentation liquid chromatography

Fatty acid methyl esters prepared from products obtained in example 1 and 2 that containing a high level of 9Z,11E,15Z-C18:3 were separated using argentation thin layer chromatography. Silica-gel plates were prepared by immersion in a 5% acetonitrile solution of $AgNO_3$ as described by Destaillats et al. (Lipids 35:1027-1032, (2000)). The developing solvent was the mixture n-hexane/diethyl ether (90:10, v/v). At the end of the chromatographic runs, the plates were briefly air-dried, lightly sprayed with a solution of 2',7'-dichlorofluorescein, and viewed under ultraviolet light (234 nm). The band at R_f = 0.52 was scraped off and eluted several times with diethyl ether. Complete evaporation of the combined was achieved with a light stream of nitrogen. The residues were dissolved in an appropriate volume of n-hexane and analysed by gas-liquid chromatography (purity superior to 98 %) as presented in example 1.

EXEMPLE 5

Preparation of mixture containing 6Z,8E,12Z- and 6Z,9Z,12Z-C18:3 acids by partial conjugation of borage oil

To commercial propylene glycol (96 g) were added NaOH (4.30 g) at room temperature. The resulting mixture was heated at 160°C for 20 min under nitrogen atmosphere and with vigorous agitation. Commercial raw linseed oil (9.35 g) was added under nitrogen atmosphere. The mixture was heated at 160°C for 1 hours under nitrogen atmosphere and with vigorous agitation. After cooling to 80°C, the reaction mixture was directly acidified with aqueous solution of H_2SO_4. After standing 10 min, the top layer was washed with a 5% NaCl aqueous solution (w/w, 47.25 kg). The top layer was removed, dried and stored at -80°C under nitrogen.

Fatty acid composition of the resulting products was performed by high resolution gas-chromatography after methylation of samples (20 mg) using the boron trifluoride method (Metcalfe et al.,). The used analytical conditions were the same as presented in exemple 1.

The obtained chromatogram was given in fig 4 and fatty acid composition of the mixture was given in table 6.

TABLE 6

Fatty Acid	% Before Reaction	% After Reaction
Palmitic	10.34	9.55
Stearic	3.36	2.38
Oleic	15.57	13.88
11Z-C18:1	0.57	0.52
Linoleic	39.96	30.11
γ-Linolenic	22.92	5.32
7,11-CCLA	0.00	1.25
9Z,11E-C18:2	0.00	6.66
10E,12Z-C18:2	0.00	6.46
9Z-C20:1	3.69	2.60
6Z,8E,12Z-C18:3	0.00	14.50
9Z-C22:1	2.05	1.22
7E,9Z,11E-C18:3	0.00	1.89

CLAIM

1. New conjugated linolenic acids.

2. Process for preparation of the conjugated linolenic acids of claim 1 comprising the steps of (a) involves blending one or a mixture of vegetable oils with various concentration of linoleic acid or partial glycerides of such oils, or partially purified or concentrated isomers with 0.5 to 10 moles of base such as sodium hydroxide, sodium alkoxylate, sodium metal, potassium hydroxide, potassium alkoxylate, potassium metal, and strong base resins (b) recovering the resulting conjugated linolenic acids.

3. The use of the conjugated linolenic acids of claim 1 as dietary supplements, food additives, cosmetic or pharmaceutical compositions formulated with conjugated products of α- and γ-linolenic acid and their related compounds used individually or in mixtures with other products.

ABSTRACT

This invention relates to new conjugated linolenic acids, process for preparation thereof and method of use. Thus the invention is concerned with the preparation and purification of fatty acids which are homologues of conjugated linoleic acids, from materials rich in alpha or gamma linolenic acids. The reaction produces approximately over one third of 9Z,11E,15Z-octadecatrienoic acid from α-linolenic acid (9Z,12Z,15Z-octadecatrienoic acid). Enrichment up to and over 40% is readily performed with urea crystallization. Moreover, the product can be produced in over 90% purity by simple preparative liquid chromatography. The reaction is unique in that the reaction produces the abovementioned conjugated trienoic acid with a high selectivity, in a short time period and in relatively mild conditions.

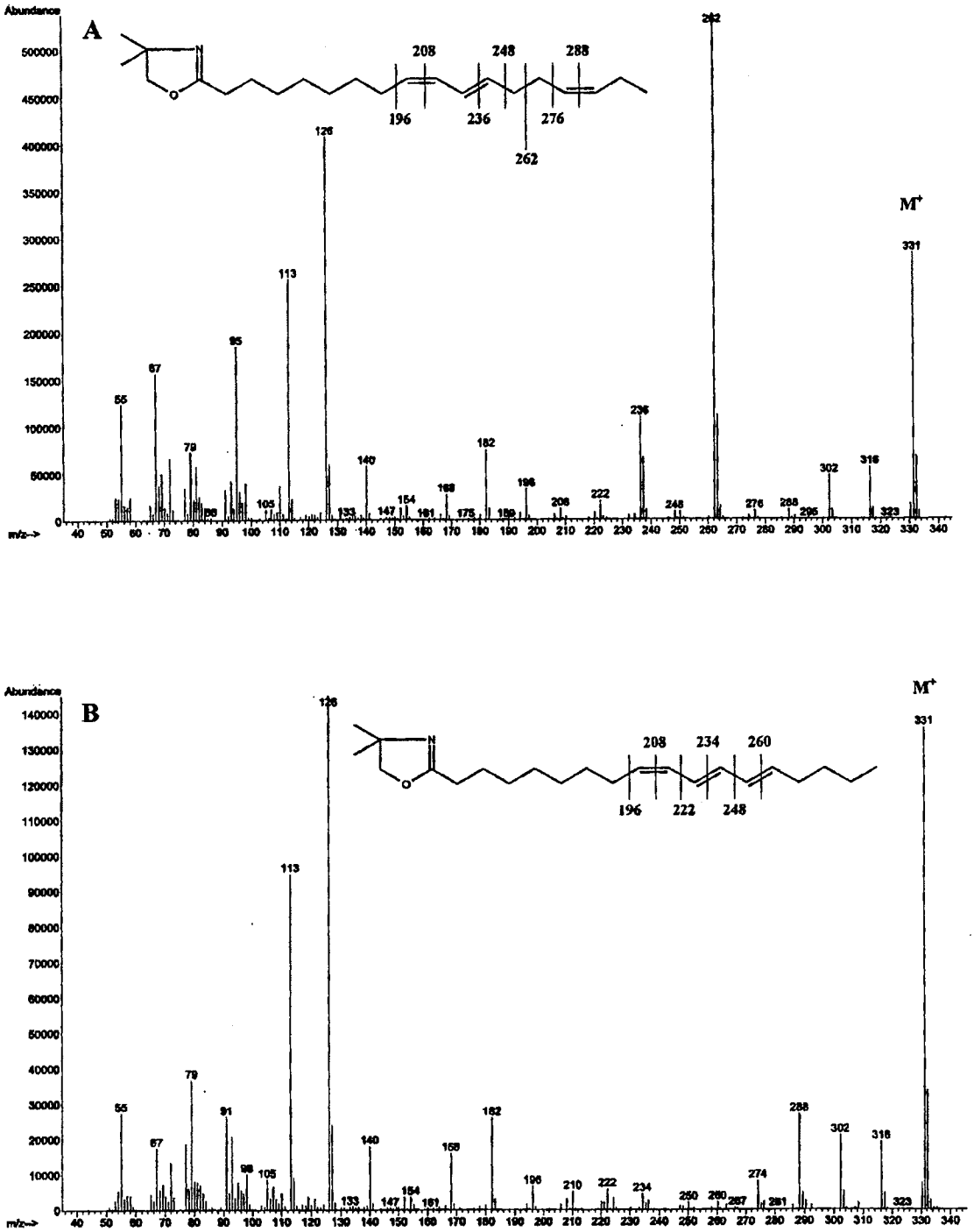
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FIG. 1

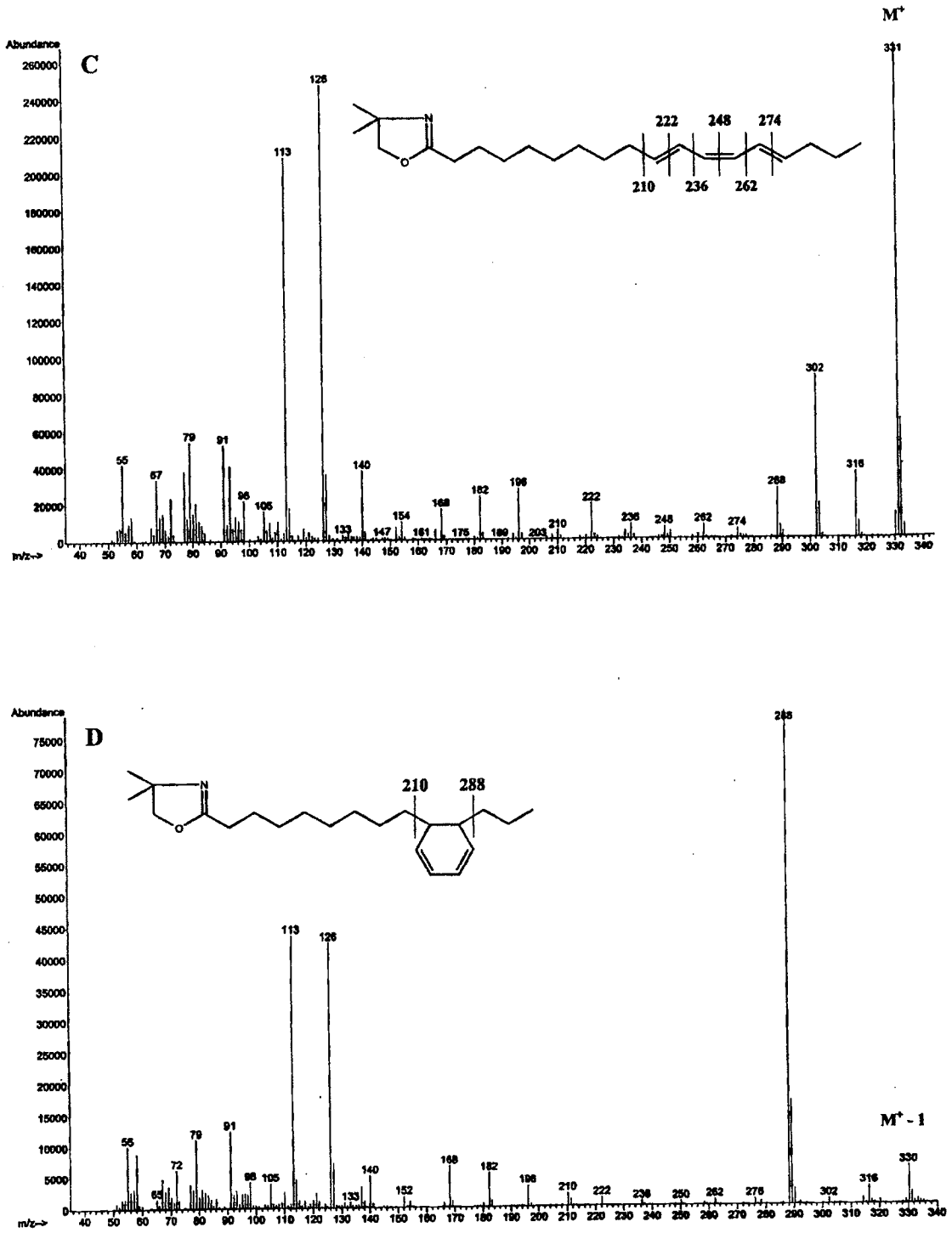


Fig. 1: presents mass spectra of produtcs resulting of the isomerization process of alpha-linolenic acid (9Z,12Z,15Z-C18:3 acid), as 4,4-dimethyloxazoline derivatives: A, 9Z,11E,15Z-C18:3; B, 9,11,13-C18:3, C, 10E,12Z,14E-C18:3 and D, 11,13-CCLA (9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid).

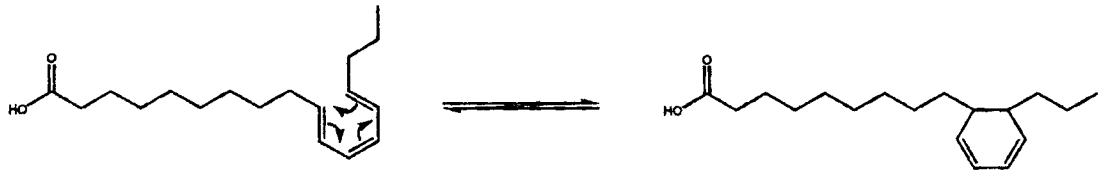
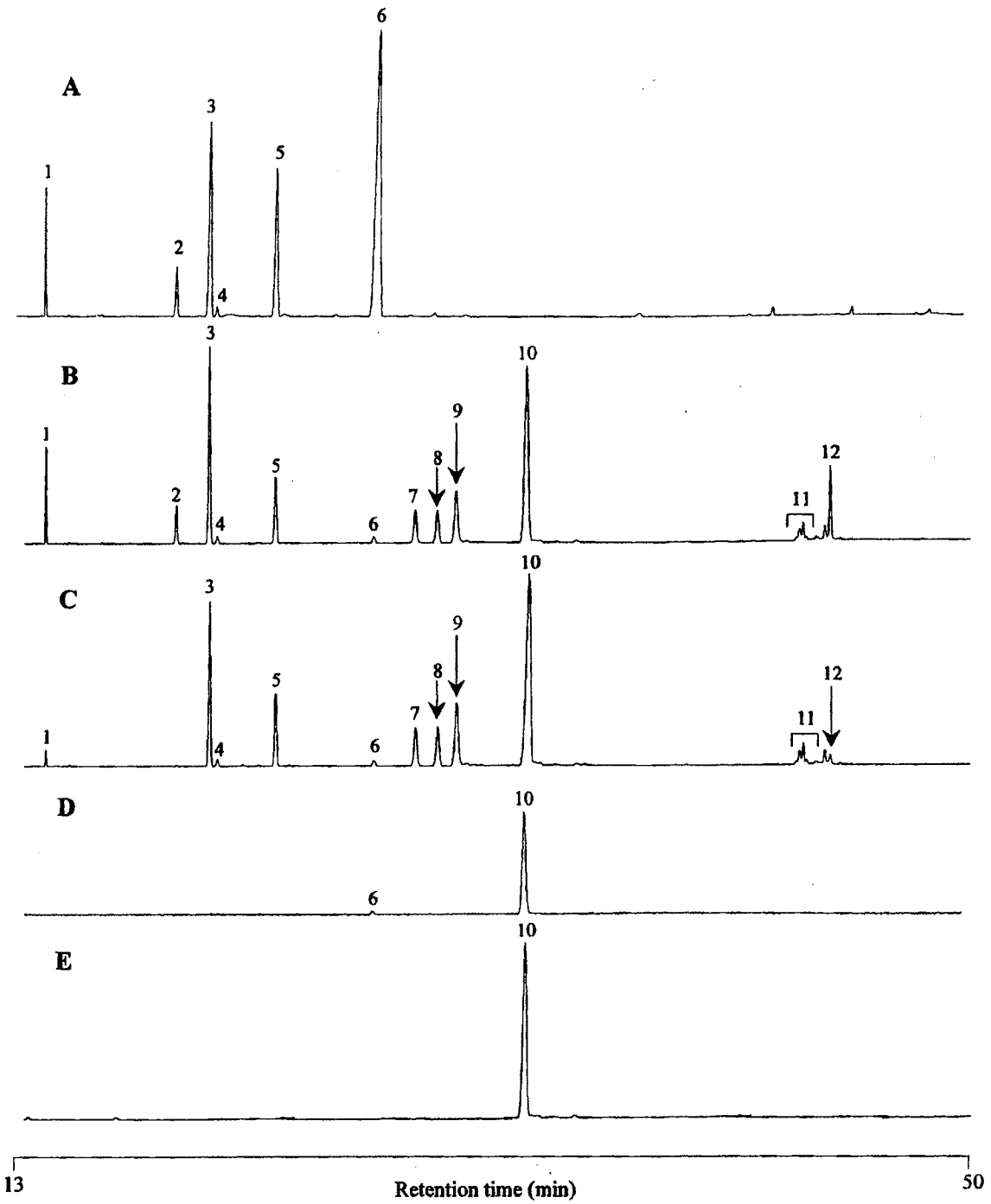
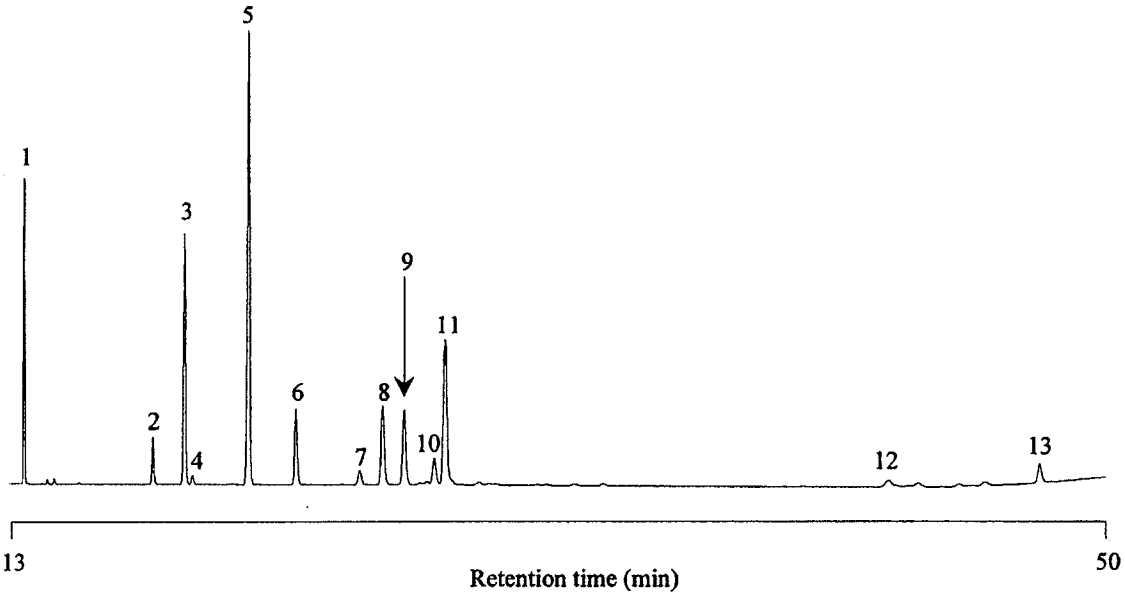


Fig. 2: present the thermal mechanism leading to the formation of 11,13-CCLA [9-(6-propyl-cyclohexa-2,4-dienyl)-nonanoic acid (Fig 1-D)] from 10E,12Z,14E-C18:3 acid.



1. Palmitic Acid
2. Stearic Acid
3. Oleic Acid
4. 11Z-C18:1 Acid
5. Linoleic Acid
6. Linolenic Acid
7. 9Z,11E-C18:2 Acid
8. 10E,12Z-C18:2 Acid
9. 9-(6-propyl-cyclohexa-2,4-dienyl)-Nonanoic Acid
10. 9Z,11E,15Z-C18:3 Acid
11. 9,11,13-C18:3 Acid
12. 10E,12Z,14E-C18:3 Acid

Fig. 3: present gas liquid chromatograms of fatty acid methyl esters obtained after methylation of linseed oil (A), conjugated linseed oil (B) liquid phase from urea crystallization (C), reversed-phase liquid chromatography fraction containing about 97 % of 9Z,11E,15Z-C18:3 acid (D), argentation liquid chromatography fraction containing about 99+ % of 9Z,11E,15Z-C18:3 acid (E)



1. Palmitic Acid
2. Stearic Acid
3. Oleic Acid
4. 11Z-C18:1 Acid
5. Linoleic Acid
6. Linolenic Acid
7. 6-(6-hexyl-cyclohexa-2,4-dienyl)-hexanoic Acid
8. 9Z,11E-C18:2 Acid
9. 10E,12Z-C18:2 Acid
10. 9Z-C20:1 Acid
11. 6Z,8E,12Z-C18:3 Acid
12. 9Z-C22:1 Acid
13. 7E,9Z,11E-C18:3 Acid

Fig 4: Gas liquid chromatogram of the fatty acid methyl esters obtained after methylation of partially conjugated evening primrose oil.